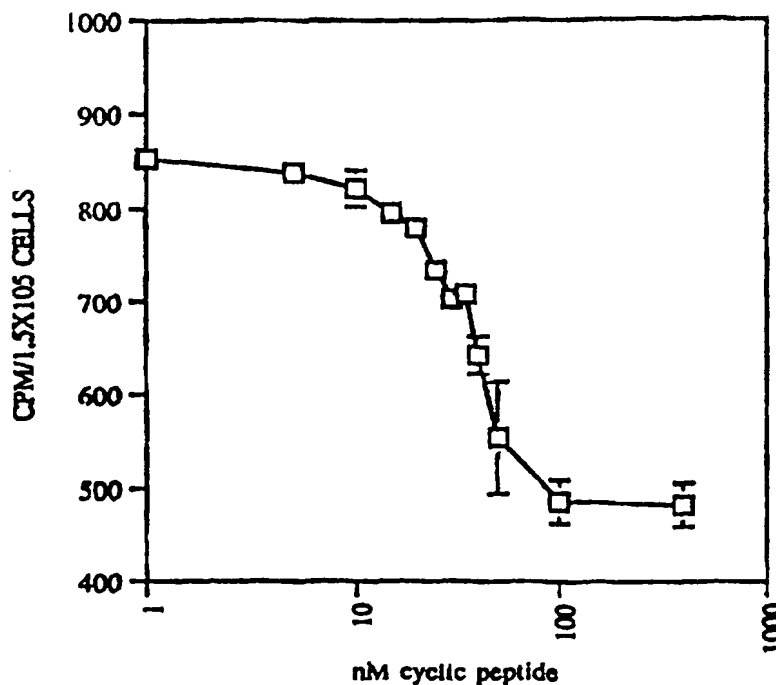# PCT

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(54) Title: METHODS FOR DIAGNOSING AND TREATING ALZHEIMER'S DISEASE

(57) Abstract

Methods for evaluating the risk of an individual to develop Alzheimer's disease using cultured neural crest-derived melanocytes are described. Also described are methods of therapy for Alzheimer's disease using peptides that bind to the neurotrophin receptor (p75^{NTR}) and competitively inhibit the binding of β-amyloid to the p75^{NTR}.

THE CYCLIC PEPTIDE COMPWTITIVELY INHIBIT β AMYLOID BINDING TO P75

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METHODS FOR DIAGNOSING AND TREATING ALZHEIMER'S DISEASE

RELATED APPLICATIONS

This application is a continuation-in-part of prior Serial No. 08/625,765 filed March 29, 1996, the teachings of which are hereby incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Dementia is a condition of deteriorating mentality that is characterized by marked decline in the individual's former intellectual level, including memory loss, impaired judgment, speech and orientation, and is often accompanied by emotional apathy. (WEBSTER'S MEDICAL DESK DICTIONARY, Merriam-Webster, Inc., Springfield, MA, p.169 (1986)).

A leading cause of dementia is Alzheimer's disease,(AD), a neurodegenerative disorder affecting 17 to 20 million people worldwide (Yamazaki, T., *et al.*, J. Cell. Biol., 129;431-442 (1995); Brinaga, M., Science, 269:917-918 (1995); Lavy-Lahad, E., *et al.*, Science, 269:970-972 (1995); Lavy-Lahad, E., *et al.*, Science, 269:973-977 (1995)). AD is characterized by progressive dementia together with neuropathological findings of "senile plaques" in the brain formed by deposits of β-amyloid protein, surrounded by clusters of degenerating neurons. β-amyloid protein itself is a fragment of the 770 amino acid membrane bound β-amyloid precursor protein (APP) that is expressed in both neuronal and non-neuronal tissues.

The specific cause of Alzheimer's disease has not yet been determined. A mutation in the βAPP gene in families with one form of autosomal dominant AD was found to be associated with increased β-amyloid synthesis and aggregation in the brain. A receptor for βAPP has been

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identified as the low density lipoprotein receptor-related protein, ApoE, and it has been postulated that this receptor protein, the enzyme responsible for βAPP cleavage in the cell membrane, production of βAPP and/or production of extracellular matrix molecules may be abnormal individually or in combination in AD patients, resulting in excess β-amyloid deposition and the observed neurotoxicity. However, the mechanism by which other known ßAPP gene mutations cause AD, as well as the pathophysiology of non-familial AD in which βAPP gene mutations have not been recognized, is not understood.

Therefore, diagnosing Alzheimer's disease as the cause of an individual's dementia is very difficult. Although recent reports of using Positron-emission tomography (PET) (Reiman, E.M., et al., New Eng. J. Med., 334:752-758 (1996), determining the genotype of an individual's ApoE, or measuring the levels of β-amyloid protein in cerebral spinal fluid may be promising, diagnosis of Alzheimer's is currently confirmed only upon autopsy to determine the presence of β-amyloid senile plaques.

In vitro systems employed to study Alzheimer's disease to date consist of malignant, or transformed, cells that are not of the same neural crest origin as neurons, or lower vertebrate neuronal cultures. It would be of great advantage to have an Alzheimer's disease model system using normal human neural crest-derived cells. However, to date, no such model system has been developed.

Moreover, recent studies have shown that damage to CNS neurons due to Alzheimer's disease begins years before clinical symptoms are evident. (Reiman, E.M., et al., New Eng. J. Med., 334:752-758 (1996)). There exists a great need for an accurate and easy to perform assay to evaluate the risk of developing Alzheimer's disease.

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SUMMARY OF THE INVENTION

The present invention relates to methods of diagnosing and treating Alzheimer's disease, and other neurodegenerative diseases mediated by β-amyloid protein, or by aberrant activation of the low affinity nerve growth factor receptor localized on neural cell surfaces. For example, autoimune encephalomyelitis, Huntington's disease, Pick's disease, corticobasal degeneration, progressive supra-nuclear palsy, Gerotman-Shausslesr Scheinker syndrome, Niemann-Pick disease, progressive supranuclear palsy are encompassed by this invention. As used herein, the term β-amyloid protein is intended to encompass β-amyloid protein (a 4.2 kD polypeptide (Selkoe, D.J., Neuron, 6:487-498 (1991); Glenner G.G. and Wong, C.W., Biochem. Biophys. Res. Commun., 120:885-890 (1993), the teachings of which are herein incorporated by reference), β-amyloid precursor protein (βAPP), and fragments of β-amyloid and β-amyloid precursor protein referred to herein as β-amyloid peptides, including β-amyloid 1-40 peptide, β-amyloid 1-42 peptide, β-amyloid 25-36 peptide or β-amyloid 28-30 peptide. (β-amyloid protein is also referred to herein as β-amyloid).

Neurodegenerative diseases mediated by β-amyloid protein encompass diseases affecting neural crest-derived cells, such as central nervous system (CNS) neurons and in which β-amyloid protein, βAPP, or β-amyloid peptides initiate, or exacerbate, a process leading to neuron degeneration, or neuronal cell death. Neurodegenerative diseases mediated by aberrant activation of the low affinity nerve growth factor encompasses diseases in which the low affinity nerve growth factor is activated by a substance other than its naturally-occurring ligand, nerve growth factor, resulting in apoptotic cell death. The neurodegenerative diseases encompassed by the present invention are characterized by progressive dementia in the

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affected individual. Specifically encompassed by the present invention is the neurodegenerative disease, Alzheimer's disease (AD), which is characterized by the deposition of β-amyloid peptides in neural tissue, leading to neuronal cell degeneration, cell death and progressive dementia.

More specifically, the present invention relates to methods of evaluating the risk of an individual to develop Alzheimer's disease using an *in vitro* assay system comprising epidermal melanocytes obtained from the individual. Epidermal melanocytes are melanocytes found in the epidermis (skin) and hair bulbs of vertebrates. The present invention is based on Applicants' findings that human melanocytes exhibit significant similarities with central nervous system neurons (the cells predominantly affected by Alzheimer's disease) and that melanocytes utilize the same signalling molecules as neurons to determine their survival versus programmed cell death (apoptosis).

For example, neuronal cells express a high affinity (p140^{TrkA}) and a low affinity receptor (p75^{NTR}) for nerve growth factor (NGF). As described herein, Applicants' have demonstrated that these nerve growth factor receptors are also expressed on melanocytes and that β-amyloid binds to the low affinity nerve growth factor receptor, p75^{NTR}, expressed on the melanocyte surface. Applicants also demonstrate herein that binding of β-amyloid to the p75^{NTR} activates the receptor, resulting in apoptotic cell death of the melanocytes. Applicants further demonstrate that the β-amyloid mediated-apoptosis can be competitively blocked by providing nerve growth factor or a biologically active fragment, analog or derivative thereof. Nerve growth factor is a physiologic ligand for p75^{NTR} that has a higher receptor affinity than β-amyloid.

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Additionally, Applicants' have demonstrated that melanocytes secrete β-amyloid (in the form of its precursor protein) constitutively, and in increased amounts in response to trauma, e.g., UV irradiation. Thus, based on results described herein, Applicants have established that melanocytes, easily obtainable from skin biopsies, are reasonable model cells for the study and diagnosis of Alzheimer's disease.

In the proposed diagnostic test, ease of induction of melanocyte apoptosis *in vitro* following exposure to β-amyloid protein and/or the blocking of this apoptosis by nerve growth factor (NGF) supplementation is correlated with the predisposition of the cell donor to develop Alzheimer's disease. Melanocytes cultivated from a patient's skin biopsy are compared to standardized control cell lines. β-amyloid protein or peptide is introduced into the cultures and melanocyte apoptosis resulting from binding of the β-amyloid protein to the $p75^{NTR}$ is determined. Activation of the $p75^{NTR}$ by binding of the $p75^{NTR}$ by β-amyloid protein, or peptide, results in apoptotic cell death of the melanocytes. Thus, typically, determination of the activation of the $p75^{NTR}$ by β-amyloid is determination of apoptotic cell death of the cultured melanocytes. Apoptotic cell death is readily assessed by any of a number of standard parameters such as propidium iodine incorporation into nuclear fragments, labelling of DNA strand breaks using fluorescein tagged dUTP in the presence of terminal deoxynucleotidyl transferase (TUNEL reaction), or by demonstration of fragmented DNA (a DNA ladder). The activation of the $p75^{NTR}$ expressed on the melanocytes obtained from the individual with the activation of the $p75^{NTR}$ expressed on the control melanocytes is then compared. If the activation of the $p75^{NTR}$ on the individual's melanocytes is greater than the activation of the $p75^{NTR}$ on the control melanocytes, it is

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indicative of the greater risk of the individual to develop
Alzheimer's disease.

 The present invention also relates to methods of
treating, or reducing the risk of developing, Alzheimer's
disease, or other neurodegenerative diseases associated
with the β-amyloid protein, or by the activation of the
p75^{NTR} resulting in apoptotic neural cell death. In the
proposed therapy for Alzheimer's disease, a composition
containing the tripeptide lysine-glycine-alanine or a
similar peptide with established affinity for the p75^{NTR}
would be delivered by an appropriate route to central
nervous system (CNS) neurons at risk of β-amyloid induced
apoptotic cell death to block βamyloid binding to p75^{NTR}.
Nerve growth factor (NGF), biologically active fragments,
analogs or derivatives of NGF (wherein biological activity
is defined herein as the ability of the fragment, analog or
derivative to bind to the p75^{NTR} expressed on neural derived
cells such as neurons and melanocytes) and/or other
neurotropins could also be administered to further compete
against β-amyloid binding to p75^{NTR}, as well as to activate
cell survival programs within damaged neurons, for example
through upregulation of the apoptosis-inhibiting protein,
Bcl-2. The therapeutic peptide is designed to have a
receptor affinity comparable to, or greater than, that of
β-amyloid but less than that of NGF and other neurotropins.

 The present invention further relates to in vitro
methods of screening substances and identifying those
substances capable of inhibiting, or decreasing neuronal
cell apoptosis mediated by β-amyloid, or activation of the
p75^{NTR}, and to substances identified by these methods.

 Alzheimer's disease is a devastating and ultimately
fatal disorder. Early detection of Alzheimer's disease
would make possible early intervention to prevent, or
substantially reduce, neuron degeneration and death.
Currently, effective therapies to treat Alzheimer's disease

are almost nonexistent. As effective treatments become available, it would permit rational therapy from an early point in the disease process when secure diagnosis by conventional clinical criteria is rarely possible. Availability of a therapeutic substance that could slow the progression of Alzheimer's disease, or other neurodegenerative diseases, in affected patients would be a great boon to those individuals.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graph depicting experimental results showing competition for binding to $p75^{NTR}$ by peptide and β-amyloid.

Figure 2 is a graph depicting experimental results of the effect of peptide on a cell survival in the presence of β-amyloid.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on Applicants' finding that melanocytes are neural crest-derived cells that share many signal transduction pathways with central nervous system neurons, including the high and low affinity receptors for nerve growth factor (Peacocke, M., et al., Proc. Natl. Acad. Sci. USA, 85:5282-5286 (1988); Yaar, M., et al., J. Cell. Boil., 115:821-828 (1991); Yaar, M., et al., J. Clin. Invest., 94:1550-1562 (1994) and basic fibroblast growth factor (Halaban, R., et al., J. Immunother., 12:154-161 (1992). Based on these significant similarities with neurons, it is demonstrated herein that cultured human melanocytes provide a model system for studies of Alzheimer's disease.

More specifically, the present invention relates to an in vitro method of evaluating the risk of an individual to develop Alzheimer's disease mediated by β-amyloid protein using cultured epidermal melanocytes obtained from the

individual. The present invention also relates to the use of the melanocyte cell culture model to evaluate therapeutic compounds effective in the treatment of Alzheimer's disease, and other neurodegenerative diseases mediated by β-amyloid protein. As described below, these methods are based on Applicants' finding that β-amyloid protein binds to the 75 kiladalton (kD) neurotrophin receptor (p75^{NTR}) (also referred to herein as the low affinity nerve growth factor receptor, or p75 nerve growth factor receptor) which is expressed on the surface of melanocytes and central nervous system neurons, and that when the p75^{NTR} is bound by β-amyloid, a pathway resulting in cell death (apoptosis) is activated.

MELANOCYTES PROVIDE A MODEL SYSTEM FOR ALZHEIMER'S DISEASE

In vitro studies have demonstrated that β-amyloid protein plays a central role in Alzheimer's disease. Attempts to understand the pathophysiology of Alzheimer's disease using malignant cell lines (Boland, K., et al., J. Biol. Chem., 270:28022-28028 (1995) or vertebrate neuronal cell cultures (Mark, R.J., et al., J. Neurosci., 15:6239-6249 (1995) have revealed that supplementation with β-amyloid peptides composed of amino acid residues 1 to 40 or 25 to 35 of the β-amyloid protein result in neurotoxicity and cell death. In particular, β-amyloid peptide comprising amino acid residues 1-40 of the β-amyloid protein, amino acid residues 1-42, or, more specifically, amino acid residues 25-35 result in neurotoxicity and neuronal cell death. Recent studies have also shown that β-amyloid-induced neurotoxicity exhibits classic features of apoptosis. The β-amyloid precursor protein (also referred to herein as βAPP) is widely expressed in neural and nonneural mammalian tissues. βAPP is most highly expressed in brain and kidney, and in the brain, neurons demonstrate particularly high expression of βAPP.

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Applicants have demonstrated that human melanocytes and cells of melanocytic origin also constitutively synthesize and secrete βAPP. After cellular trauma such as ultraviolet irradiation, there is increased βAPP secretion,
5   leading to increased βAPP concentration in the vicinity of cultured cells. (Andersen, W., et al., J. Invest. Dermatol., 104:585 Abst.182 (April 1995).

Applicants demonstrate herein that supplementation of normal human melanocytes with β-amyloid 1-40 peptide at low
10   concentrations (≤1μM) leads to extensive outgrowth of dendrites, the melanocyte cell processes analogous to neurites of neurons, without decreasing cell yields. At higher concentrations of β-amyloid peptide, melanocyte cell yields decrease progressively and the remaining cells
15   appear unhealthy. Additionally, in these cultures there is focal development of plaque-like structures consisting of aggregated dying melanocytes, similar to the in vivo "senile plaques" observed in patients with AD.

Applicants further demonstrate herein that maintaining
20   melanocytic cell cultures in ≥25 μM β-amyloid 1-40, compared to control cultures, significantly increases the proportion of apoptotic cells and upregulates Bax protein expression approximately three fold. Recent in vivo and in vitro data suggest that the β-amyloid induced-neuronal
25   death exhibits classical characteristics of programmed cell death or apoptosis (Cotman, C.W. and Anderson, A.J., Mol. Neurobiol., 10:19-45 (1995); Su, J.H., et al., Neuroreport, 5:2529-2533 (1994). The molecular pathways that regulate apoptosis in neurons have been identified in part.
30   Evidence suggests that the product of the proto-oncogene Bcl-2 delays the onset of apoptosis in neurons that are dependent for survival on neurotrophic factors (Allsopp, T.E., et al., Cell, 73:295-307 (1993); Garcia, I., et al., Science, 258:302-304 (1992). Conversely, overexpression of
35   a 21 kD Bcl-2-associated protein, Bax, accelerates

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apoptotic death of cells (Oltvai, Z.N, et al., Cell, 74:609-619 (1993)).

Melanocytes express both the low affinity p75[NTR] and high affinity 140 kD trk A (p140[trkA]) receptors for NGF and providing NGF to melanocytes results in activation of the p140[trkA] pathway, presumably through co-ordinate binding of p140[trkA] and multiple p75[NTR] molecules as postulated to occur in neurons exposed to NGF, that in turn activates an intracellular signal transduction pathway leading to enhanced expression of Bcl-2 and enhanced cell survival.

Applicants further demonstrate herein that βamyloid binds competitively to the p75[NTR]. Studies previously published suggest that the specific binding site for the p75[NTR] is amino acids 29-36 of the resulting NGF protein (Ulrich, A., et al., Nature 303:821-825 (1983), and that if the sequence lysine-glycine-lysine (residues 32-34 of NGF) is changed to lysine-glycine-alanine, the peptide has approximately half the affinity for the receptor as native NGF. Amino acid residues 28-30 of the β-amyloid protein are lysine-glycine-alanine. Furthermore, computerized structure analysis of βamyloid reveals that these amino acids have a high probability of being in a loop turn of the protein, suggesting a high probability that this β-amyloid peptide sequence plays a role in receptor binding.

A cyclic decapeptide was therefore synthesized by attaching two cysteine residues to the beginning and the end of the β-amyloid fragment consisting of amino acids 24-31: VGSNKGAI (SEQ ID NO: 1). Cold peptide competitively inhibited ^{125}I-β-amyloid binding, with 50% inhibition occurring at 25 nM. Furthermore, β-amyloid 200 nM reduced by cell yields by ~60% (p<.02), but this cell loss was blocked by the peptide (200 nM). Peptide alone had no effect on cell yield. These findings indicate that apoptosis of neurons in Alzheimer's Disease results from the interaction of β-amyloid with p75[NTR]. The data also

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suggest that β-amyloid-mediated death of neurons may be
prevented by delivery of a synthetic peptide that blocks
the β-amyloid binding sites.

Thus, based on the above findings on significant
similarities between CNS neurons and epidermal melanocytes,
permitting their use in diagnostic testing and drug
development studies for Alzheimer's disease.

IN VITRO ASSAY FOR ALZHEIMER'S DISEASE

The melanocyte model system described herein can be
used for the early identification of individuals who are at
risk for developing Alzheimer's disease. At present, there
are three known or suspected mechanisms for the development
of Alzheimer's disease: 1.) Increased production and/or
secretion of β-amyloid by CNS neurons, a dysfunction known
to result from one of the recognized βAPP mutations in
familial Alzheimer's disease patients; 2.) Increased
sensitivity to physiologic β-amyloid concentration due to
excessive local aggregation of the β-amyloid peptide for as
yet unknown reasons, speculated to result from subtle
structural alterations in the secreted β-amyloid or from
subtle abnormalities of the extracellular matrix in the
CNS; and 3.) Decreased expression or function of the low
density lipoprotein receptor-related protein on the surface
of neurons that is known to bind, internalize, and degrade
βAPP and whose functional deficiency would result in
increased amounts of extracellular β-amyloid.

Melanocytes are isolated from skin biopsies obtained
from the individual to be tested, i.e., test melanocytes.
Skin biopsies are performed using standard dermatological
techniques. Typically, after application of a local
anesthetic, a 3-4 mm punch biopsy is obtained from the skin
of the individual. Any convenient skin site for biopsy can
be selected. Melanocytes isolated from skin are also
referred to herein as epidermal melanocytes.

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The melanocytes obtained from the individual are cultured under standard laboratory conditions as described herein, typically using serum-free medium. Control cultures of melanocytes are also maintained under similar conditions. Control melanocytes can be obtained from an individual known to be disease-free, from neonatal foreskins, or from available melanocyte cell lines. (See, e.g., Park, H-Y. et al., J. Biol. Chem., 268:11742-11749 (1993)). All melanocytes express the p75^{NTR} plus p140$^{trk\ A}$ receptor proteins. The cultures are maintained under these conditions for approximately two days to ensure stable, viable melanocyte cultures. The cultures can be maintained for longer time periods as long as the melanocytes remain viable.

After culture stabilization β-amyloid protein or β-amyloid peptide is introduced into the cultures. As defined herein, β-amyloid protein includes β-amyloid precursor protein as well as β-amyloid protein. β-amyloid peptide includes β-amyloid 1-40 peptide; β-amyloid 1-42 peptide; β-amyloid 25-36 peptide and β-amyloid 28-30 peptide. β-amyloid proteins and peptides are commercially available from various sources, e.g, Bachem California, Torrance, CA. β-amyloid protein and peptides can also be chemically synthesized or recombinantly produced using known laboratory techniques. The β-amyloid protein or peptide introduced into the culture is typically dissolved in a buffer compatible with the cell culture medium. The concentration of β-amyloid protein or peptide added to the culture can vary from 0μM to 100 μM, typically from 1μM to 50 μM. A typical single dose of β-amyloid protein or peptide is approximately 25 μM. The concentration of β-amyloid protein or peptide added is a concentration sufficient to bind to, and activate the p75^{NTR}.

As defined herein, activation of the p75^{NTR} by β-amyloid protein or peptide means the initiation, or

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activation of the apoptotic cell death pathway in the
melanocyte. As further defined herein, the activation of
the p75^{NTR} also means the induction of Bax protein or the
initiation of sphingomyelin hydrolysis.

5   The melanocyte cultures are maintained in the presence
of β-amyloid protein or peptide for a time sufficient for
detectable activation of the p75^{NTR} to occur, typically
about three days. However, the time can be as short as 1
or as long as 8 days. The activation of the p75^{NTR} of the
10   test melanocytes is compared with the activation of the
p75^{NTR} of the control melanocytes.

        The activation of the p75^{NTR} by β-amyloid results in
apoptotic cell death of the melanocytes. Apoptosis is
determined using well-known laboratory techniques. As
15   described herein in Example 1, an assay to determine
melanocyte cell yield was used to determine apoptosis.
Apoptosis decreases cell yield. Cell yield is determined
by counting viable cells, such as with an electronic cell
counting instrument (e.g., a Coulter^{TM} cell counter) or by
20   performing a manual cell count using a hemocytometer.

        Also as described in Example 1, the cultures can be
microscopically evaluated for the presence of plaque-like
structures containing dying, and/or dead melanocytes.
These plaques also contain β-amyloid deposits.

25   As described in Example 2, activation of the p75^{NTR} can
also be determined using an assay to measure the inducement
of Bax protein expression. Bax is the Bcl-2-associated
protein implicated in the apoptotic cell death pathway. An
increase in the expression of Bax is an indication of
30   apoptosis. Measurement of Bax protein expression can be
accomplished by determining the increase of Bax mRNA
expressed in the cells, using standard laboratory
techniques to determine RNA in cells. Bax inducement can
also be measured using anti-Bax antibody in Western blot
35   analysis.

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Apoptosis can also be determined by the measurement of propiduim iodine incorporation into nuclear fragments, the TUNEL reaction or demonstration of fragmented DNA.

If the activation of the p75^{NTR} of the test melanocytes is greater than the activation of the p75^{NTR} of the control melanocytes, it is an indication that the individual from whom the test melanocytes were obtained has neural crest cells that are more sensitive than standardized control cell lines to β-amyloid. If the individual's neural crest cells are more sensitive to β-amyloid, it is reasonable to predict that the individual is at risk to develop Alzheimer's disease.

In individuals whose melanocytes exhibit abnormal sensitivity to β-amyloid, further characterization of the synthesis and deposition of extracellular matrix molecules can be performed using the melanocyte culture method to determine the interaction of these extracellular molecules with the β-amyloid peptide.

The cultured melanocyte assay method described herein can also be useful to evaluate the function of the low density lipoprotein receptor-related protein on the patient's neural crest cells.

Neurons constitutively secrete β-amyloid precursor protein. Because β-amyloid is a major component of the hallmark senile plaques found in Alzheimer's disease patients, the presence of increased levels of β-amyloid protein and/or peptide surrounding neurons is reasonably correlated with the presence of Alzheimer's disease. Using the melanocyte culture assay method described herein, it can be determined if the patient's neural crest cells synthesize and/or secrete increased levels of β-amyloid constitutively or in response to trauma, in greater amounts as compared to standardized control cell lines. If the secretion of β-amyloid protein or peptide is greater in the test melanocyte culture than in the control melanocyte

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culture it is indicative that the individual has an
increased risk of developing Alzheimer's disease.

As described above, a number of pathologic mechanisms
leading to Alzheimer's disease are known or suspected. As
described herein, Applicants demonstrate that the final
pathway upon which all the above mechanisms converge is the
activation of the p75^{NTR} alone, leading to apoptosis,
instead of the beneficial activation of a receptor complex
consisting of NGF, p75^{NTR} and p140$^{trk A}$. Therefore, the
determination of the individual's risk developing
Alzheimer's disease can be accomplished by measuring levels
of p75^{NTR} versus p140^{trkA} expressed on the surface of the
individual's melanocytes. Individuals with a high ratio of
p75^{NTR} to p140$^{trk A}$ would be those at risk for developing
Alzheimer's disease.

Thus, using the melanocyte culture methods described
herein, it is reasonable to predict whether an individual
is at risk to develop Alzheimer's disease. Moreover, using
the melanocyte culture methods it is also reasonable to
predict by what mechanism given individuals are likely to
develop the disease, e.g., abnormal sensitivity βamyloid,
increased secretion of β-amyloid protein, or abnormal ratio
of expressed p75^{NTR} to p140^{trkA}, and thus allow selection of
a therapeutic approach that specifically targets the
abnormality. Furthermore, the melanocyte culture methods
of the present invention are useful to screen therapeutic
substances, as described below, to determine their
effectiveness in an individual. Thus, an individual with
either a known predisposition to Alzheimer's disease, or a
diagnosis of Alzheimer's disease based on conventional
clinical criteria, will be treated with a substance of
demonstrated in vitro efficacy, and thus, increase chances
of alleviating the disease symptoms.

EVALUATION OF EFFECTIVE ALZHEIMER'S DISEASE THERAPIES

NGF binds two receptors: the protein product of the trkA proto-oncogene, p140^{trkA}, a transmembrane tyrosine kinase receptor, and a 75 kD low affinity transmembrane receptor for several neurotrophins, p75^{NTR}. p140^{trkA} has been reported to mediate NGF-induced effects in the absence of p75^{NTR} (Verdi, J.M., et al., Proc. Natl. Acad. Sci. USA, 91:3949-3953 (1994)). It has also been reported that p75^{NTR} mediates apoptosis of developing neurons in the absence of trkA and that modulation of p75^{NGF} receptor can promote neuronal survival (Catharina, F.E., Science, 1725:1729-1732 (6 December 1996)). However, the functional importance of p75^{NTR} in NGF signal transduction is still controversial. It was reported that co-expression of p75^{NTR} and p140^{trkA} produces functional high-affinity binding of NGF (Battleman, D.S., et al., J. Neurosci., 13:941-951 (1993)). However, it was also reported that activation of p75^{NTR} in cells that do not express p140^{trkA} induces their apoptosis by activation of the sphingomyelin signalling pathway. It is therefore possible that p75^{NTR} has a dual role. In combination with p140^{trkA} it may signal through a tyrosine kinase-dependent pathway (Dobrowsky, T.T., et al., Science, 265:1596 (1994) leading to survival, but when activated alone it signals through the sphingomyelin pathway leading to apoptosis.

Binding of NGF to p75^{NTR} is mediated through amino acid residues 29-36, TDIKGKEV (SEQ ID NO: 2), that are part of the β-hairpin loop of NGF (Ibánez, C.F., et al., Cell, 69:329-341 (1992)). If lysine (K) at position 34 is replaced by alanine (A), the resulting mutant NGF molecule still binds p75^{NTR} but with 50% lower affinity. Interestingly, in β-amyloid the amino acid residues 28-30, which are present in both the 1-40 and the 25-35 β-amyloid peptides, are KGA, a sequence that appears to permit p75^{NTR} binding by β-amyloid. Computerized structure analysis of

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β-amyloid suggests that the KGA residues have a high probability (>60%) of being in a loop turn, the highest probability of any portion of this 40 amino acid peptide, suggesting that this sequence constitutes a binding site for p75^{NTR}. Furthermore it was reported that expression of p75^{NTR} enhances the toxic effect of β-amyloid on cells, possibly through binding and activation of the receptor.

Based on the above data, it is reasonable to believe that the specific three amino acid sequence lysine-glycine-alanine (KGA) in the β-amyloid protein binds the 75 kD transmembrane neurotrophin receptor on CNS neurons, activating the programmed cell death pathway, mediated in part by an increase in intracellular Bax levels.

It is also reasonable to believe that competitively inhibiting the binding of β-amyloid peptide blocks this aberrant receptor activation and the resulting apoptosis. For example, providing full-length NGF, or a biologically active fragment, analog, derivative, variant or mutant thereof results instead in preferential binding of p75^{NTR} coordinately with p140^{trkA} binding, leading to activation of a second signal transduction pathway that results in neuronal cell survival.

The term "biological activity" of NGF, or a fragment, derivative, analog, variant or mutant NGF, is defined herein as the activity of the NGF to specifically bind to the p75^{NGF} receptor. Such activity can be measured by the methods described herein, or by other methods known to those skilled in the art. Another biological activity of an NGF fragment, analog, derivative, variant or mutant is the antigenic property of inducing a specific immunological response as determined using well-known laboratory techniques. For example, a biologically active NGF fragment can induce an immunological response which produces antibodies specific for the NGF (anti-NGF antibodies).

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An "analog" is defined herein to mean an amino acid sequence with sufficient identity to amino acid sequence of the endogenous NGF to possess the biological activity of the protein. For example, an analog of a polypeptide can have introduced into the polypeptide amino and sequence "silent" changes wherein one or more amino acid residues differ from the amino acids residues of the NGF yet still possess P75^{NTR} binding activity. Examples of such differences include additions, deletions, or substitutes of residues. Also encompassed by the present invention are proteins that exhibit lesser or greater biological activity of NGF.

The present invention also encompasses the production and use of biologically active fragments of the NGF described herein. Such fragments can include only a part of the full length amino acid sequence of NGF, yet possess biological activity. As used herein, the term "biologically active fragment" means NGF a fragment that can exert a biological or physiologic effect of the full-length protein, or has a biological characteristic, e.g., antigenicity, of the full-length protein. Such activities and characteristics are described herein. Such fragments can be produced by amino and carboxyl terminal deletions as well as internal deletions. Also included are active fragments of the protein, for example, as obtained by enzymatic digestion. Such peptide fragments can be tested for biological activity.

"Derivatives" and "variants" of NGF is NGF which has been modified. This includes NGF which have been modified by alterations in the amino acid sequence associated with the p57^{NTR}. This also includes, but is not limited to, truncated and hybrid forms of NGF. "Truncated" forms are shorter versions of NGF, for example, modified so as to remove the C-terminal regions. "Hybrid" forms include NGF

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that is composed of portions of two or more proteins, i.e.,
a fusion protein comprising NGF with another protein.

Variants can be produced using the methods known to
those of skill in the art. The NGF gene can be mutated *in
vitro* or *in vivo* using techniques known in the art, for
example, site-specific mutagenesis and oligonucleotide
mutagenesis. Manipulations of the NGF sequence can be made
at the protein level as well. Any of numerous chemical
modifications can be carried out by known techniques
including, but not limited to, specific chemical cleavage
by cyanogen bromide, trypsin, and papain. It can also be
structurally modified or denatured, for example, by heat or
by being immobilized on a solid surface.

The amino acid sequences of the NGF fragments,
analogues, derivatives, variants and mutants of the present
invention can be altered to optimize NGF binding to $p75^{NTR}$,
by methods known in the art by introducing appropriate
nucleotide changes into native or variant DNA encoding the
NGF, or by *in vitro* synthesis of the desired NGF.

It is reasonable to assume that NGF has a higher
affinity for the $p75^{NTR}$ than does β-amyloid, and that when
NGF is present in the cellular environment, this
neurotrophin preferentially binds the $p75^{NTR}$ coordinately
with the $p140^{trkA}$ receptor, activating $p140^{trkA}$ and leading to
cell survival. However, under conditions leading to
increased surface expression of $p75^{NTR}$ or increased levels
of β-amyloid in the extracellular space, in the absence of
saturating amounts of NGF, apoptotic cell death may result
from binding of β-amyloid to the $p75^{NTR}$ with subsequent
activation of the sphingomyelin pathway. In the CNS, such
a situation might occur in older individuals, particularly
those with a genetically-determined excessive production of
β-amyloid fragments containing the amino acid sequence
required for $p75^{NTR}$ binding. Normal human melanocytes
appear to respond to environmental signals in the same

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manner as CNS neurons and reasonably provide a model system for studying potential therapies for Alzheimer's disease.

Thus, the present invention also encompasses methods employing the cultured melanocyte model system for the development and evaluation of substances to enhance cell survival and to block β-amyloid-induced apoptotic cell death. Specifically, the methods of the present invention can be used to identify and evaluate substances to treat Alzheimer's disease. For example, the melanocyte culture model can be used to identify substances that block the p75NTR binding of β-amyloid, and thus block β-amyloid-mediated neuronal apoptosis. As described above, the tripeptide lysine-glycine-alanine is a candidate substance for use as a therapeutic to alleviate the symptoms of Alzheimer's disease. The melanocyte culture model system can be used to identify and evaluate other peptides containing the KGA sequence, or various analogs of the KGA tripeptide, to determine an optimum composition that will bind p75^{NTR} and thus block the binding of the apoptosis-inducing β-amyloid ligand without interfering with NGF binding that is beneficial. Because evaluation of candidate substances is made with melanocytes obtained specifically from the individual at risk, or with the diagnosis of AD, the probability of identifying an effective candidate substance is very high.

Once candidate substances are identified, the therapeutic level of substance that must be delivered to the CNS can be accurately determined using *in vitro* melanocyte cultures from each individual. Titration of concentration of the candidate substance can be performed using the melanocyte culture model system described herein.

Also encompassed by the present invention are methods of therapy for the treatment of neurodegenerative diseases resulting from apoptosis due to the aberrant activation of the p75^{NTR} receptor of neural crest-derived cells.

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Specifically encompassed are methods of therapy for individuals experiencing symptoms of dementia resulting from degeneration, and death, of neurons due to β-amyloid-mediated apoptosis. β-amyloid-mediated apoptosis is a

5   hallmark of Alzheimer's disease, therefore, also specifically encompassed by the present invention are methods of treating individuals with Alzheimer's disease.

      Methods of therapy include administering to the individual a substance, e.g., the tripeptide KGA, or an

10   analog thereof, in a manner which permits contact of the substance with neurons of the CNS. For example, the pentapeptide CKGAC (SEQ ID NO: 3), or an analog thereof, can be chemically synthesized by methods well-known to one of skill in the art. The cysteine residues flanking the

15   ends of the pentapeptide can be linked, e.g., by a disulfide bond, to maintain the conformation required for binding of the peptide to the p75^{NTR}, thus inhibiting, or preventing apoptosis. The length of the peptide can be longer than a pentapeptide, as long as the KGA, or analog

20   peptide is maintained in a configuration suitable for binding activity. For example, as described herein, cyclic peptides have been made with the amino acid sequences and CVGSNKGAIC (SEQ ID NO: 4) these peptide compete for p75^{NTR} binding with β-amyloid peptide.

25   Administration, or delivery of the peptide, or other substance, can be accomplished in a manner similar to methods used in gene transfer and therapy. For example, an effective amount of the DNA encoding the peptide can be inserted into a virus vector construct which targets

30   central nervous system neurons. The DNA insert also includes sequences necessary for the expression of the DNA in the target cells. Particularly useful is the Herpes Simplex Virus-1 (HSV-1) vector described in Geller, A.I., and Breakefield, X.O., _Science_ 241:1667-1669 (1988) and

35   U.S. Patent 5,288,641 (Roizman 1994), the teachings of

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these references are herein incorporated by reference.
Gene guns can also be used. Additionally, intracranial
administration can also be employed.

Further in vivo testing cam be performed using an art-
recognized mouse model such as the transgenic mouse
described in WO 96/40895, the teachings of which are
incorporated herein by reference. Such testing is well-
known to those of skill in the art.

The following examples more specifically illustrate
the invention and are not intended to be limiting in any
way.

EXAMPLE 1:   THE EFFECT OF β-AMYLOID ON NORMAL HUMAN
MELANOCYTES

To determine the effect of β-amyloid on normal human
melanocytes,   cultures were supplemented with increasing
concentrations (0.025 - 50 uM) of HPLC-purified β-amyloid
fragment corresponding to amino acids 1-40. A peptide
containing the first 40 amino acids synthesized in reverse
order (40-1) was used as a negative control.

Melanocytes were maintained in serum free Medium 199
(Gibco BRL Gaithersburg, MD) supplemented with epidermal
growth factor (10 ng/ml) (Collaborative Research), insulin
(10 μg/ml) (Sigma), triiodothyronine (10^{-9} M)
(Collaborative Research), transferrin (10 μg/ml) (Sigma),
hydrocortisone (1.4 x 10^{-6} M) (Calbiochem), cholera toxin
(10^{-9} M) (Calbiochem) and basic fibroblast growth factor
(basic FGF) (10 ng/ml) (Collaborative Research). Two days
after plating cells were supplemented with increasing
concentrations of β-amyloid 1-40 or the control peptide 40-
1 (0-50 μM) (Bachem California, Torrance, CA). Cell yields
determined three days after addition of β-amyloid show a
dose dependent decrease of cell yield in cultures
maintained with the 1-40 peptide. No effect on cell yield

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was observed in cultures maintained with the control 40-1 peptide.

Melanocytes maintained in the presence of 25-30 uM β-amyloid 1-40 showed a 59% ± 17% decrease in cell yield as compared to cell yield before β-amyloid addition that is considered to be 100%. Cell yield of duplicate cultures maintained in the presence of the control 40-1 peptide showed 8% ± 32% increase in cell yield (p<0.02, paired t test).

Melanocytes maintained in the presence of the 40-1 control peptide have a typical bipolar to polygonal morphology. The majority of the melanocytes maintained in the presence of the 1-40 peptide are rounded and detaching from the dish surface.

Regression analysis showed significant decrease in cell yield with increasing concentrations of β-amyloid 1-40 (R^2=0.8475, p<0.00001) but no significant effect on cell yield of β-amyloid 40-1 (R^2=0.06, p=0.44). In a total of four experiments, within 3-5 days β-amyloid 1-40 decreased melanocyte yield by >50% (p<0.02; paired t test) while the control 40-1 β-amyloid peptide at the same concentrations had no effect on cell yield.

EXAMPLE 2: EFFECT OF β-AMYLOID 1-40 ON MELANOCYTE PLAQUE FORMATION

Melanocyte cultures, cultured as described above in Example 1, were also evaluated for plaque formation. In some cultures the development of plaque-like structures consisting of increasingly large congregations of dying melanocytes was noted, reminiscent of the senile plaques described in the brains of patients with Alzheimer's disease.

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EXAMPLE 3: EFFECT OF β-AMYLOID AND NGF ON MELANOCYTES

In neurons, the protein product of the proto-oncogene Bcl-2 delays the onset of apoptosis triggered by a variety of stimuli, while overexpression of a Bcl-2 associated protein (Bax) accelerates this cell death.

To investigate the mechanism of β-amyloid-mediated melanocyte death, Bax levels in melanocytes treated with 25 uM of 1-40 or 25-35 β-amyloid peptides was examined. Within 4 days of treatment, Bax was induced 3 fold in melanocytes stimulated either with the β-amyloid 1-40 or 25-35 fragments as compared to melanocytes treated with the 40-1 control fragment or an irrelevant HPLC purified protein of similar size.

Melanocytes were maintained as above. Four days after addition of 25 μM of β-amyloid fragments 1-40 40-1 or 25-35; or 25 μM of HPLC-purified bovine corticotropin releasing factor (CRF) (Bachem California) (MW 4.7 kD) as an additional negative control, cells were extracted in RIPA buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100) in the presence of 1 μg/ml aprotinin and 75 μg/ml phenylmethylsulfonyl fluoride (PMSF), sonicated for 1-3 seconds and centrifuged. 40 μg of protein per lane were separated on 12% SDS/PAGE and blotted onto nitrocellulose paper (overnight, 25V). To verify equal loading a duplicate 13% SDS/PAGE was performed and stained with Coomasie Blue R250 stain. Blots were incubated with anti-Bax antibody (1:1000 dilution) (primary antibody), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (secondary antibody) (1:500 dilution) (Bio-Rad Laboratories, Hercules, CA). Bound antibodies were detected using enhanced chemiluminescence kit (Amersham Corp.). Autoradiograms were scanned into a Macintosh II is computer using the Ofto TM program (Light source Computer Images, Inc.). Scan analysis was performed

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with manual definition of bands using Scan Analysis TM
68000 program (Biosoft, Cambridge, UK).

5
        β-amyloid peptides 1-40 and 25-35 increased Bax level
as compared to the 40-1 peptide (270% and 160%,
respectively).

EXAMPLE 4: EFFECT OF NGF ON β-AMYLOID INDUCED CELL DEATH
        Nerve growth factor (NGF) has been reported to improve
cognitive function and attenuate loss of cholinergic
10
neurons in animal models and in clinical treatment trials
for Alzheimer's disease (cto, A., et al., Behav. Brain.
Res., 57-255-261 (1993); Lapchak, P.A., Exp. Neurol.,
124:16-20 (1993); Olson, L., et al., J. Neural. Transm.
Park. Dis. Dement. Sect., 4:79-95 (1992)). Furthermore, it
15
has been recently reported that NGF delays melanocyte
apoptosis by upregulating the levels of Bcl-2 (Zhai, S., et
al. Exp. Cell. Res.. It was investigated whether NGF
supplementation protects melanocytes from β-amyloid-induced
cell death. Addition of NGF to β-amyloid-supplemented
20
melanocytes enhanced cell yields within 3-5 days and
greatly improved the morphology of surviving cells in most
donors, although degree of protection was variable among
donors. Preliminary data suggest that NGF supplementation
decreases the β-amyloid-induced Bax upregulation and
25
increases Bcl-2 levels in the cells suggesting that NGF
interferes with β-amyloid-mediated signal transduction.
        Melanocytes were maintained as above in hormone
supplemented medium lacking hydrocortisone. Cells were
supplemented with 25 μM of β-amyloid 1-40 in the presence
30
of 50 ng/ml NGS or diluent.
        Photographs of representative fields were obtained and
the percent of the live cells (spread) was determined 48
hours after addition of β-amyloid and NFG or β-amyloid and
diluent. In the presence of β-amyloid and diluent 77$\pm$8.5%
35
appeared spread as compared to 96$\pm$1.4% in cultures

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supplemented in β-amyloid and NGF. In cultures not
provided β-amyloid and diluent even the cells that were
still spread on the dish surface were vacuolated and
generally did not appear as healthy as cells in NGF

5   supplemented cultures.

β-amyloid exposed melanocytes in the absence of NGF
are dying, while in the presence of NGF the cells appear
healthy and spread on the dish surface. At least 400 cells
were counted in each condition.

10

EXAMPLE 5: β-AMYLOID BINDS THE p75 NERVE GROWTH FACTOR
RECEPTOR

To determine if β-amyloid binds p75^{NTR}, ^{125}I 1-40 β-
amyloid peptide was added to permanently transfected

15   cultured fibroblasts that overexpress p75^{NTR} (p75^{NTR}-NIH 3T3
cells) (Dobrowsky, T.T., et al., Science, 265:1596 (1994)
in the presence of disuccinimidyl suberate to cause cross
linking of closely associated proteins. Cells were then
immunoprecipitated with anti p75^{NTR} antibodies or an

20   irrelevant mouse IgG. Autoradiograms revealed a protein
band of 75-80 kD in size only in lysates immunoprecipitated
with anti p75^{NTR} antibodies. Competition analysis of ^{125}I 1-
40 β-amyloid in the presence of increasing concentrations
of unlabeled NGF showed that 1-40 β-amyloid could be

25   competed off by NGF. However, residual ^{125}I 1-40 binding
suggests that 1-40 β-amyloid may have an additional cell
surface receptor, perhaps the recently identified serpin-
enzyme complex receptor.

Results indicate that p75^{NTR} is a receptor for β-

30   amyloid, a peptide that is reported to be secreted into the
medium of normal cells in high picomolar to low nanomolar
concentrations.

p75^{NTR}-NIH 3T3 cells were maintain in DMEM supplemented
with 10% FBS in the presence of penicillin (45 ng/ml),

35   streptomycin (68 ng/ml), and ihygromycin B (17.5 ng/ml).

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At 80% confluence cells were lifted from the dish with EDTA
and incubated in suspension with 5 uCi ^{125}I β-amyloid 1-40
at 4°C for 1 hour in DMEM. After incubation, 1 mM of
disuccinimidyl suberate was added for 30 minutes.
5   Following centrifugation cells were lysed with RIPA buffer
(50 mM Tris HCl, pH 8.0, 0.15 M NaCl 0.5% sodium
deoxycholate 4, 5 mM MgC$_2$, 1% Triton x-100, 1 mM
phenylmethylsulfonylfluoride [PMSF], and 1 μg/ml
aprotinin), sonicated for 1-3 seconds and
10   immunoprecipitated with anti p75^{NTR} antibodies (mouse
monoclonal IgG1, Cedarlane Laboratories Ltd., Ontario,
Canada) or mouse IgG as control for 16 hours at 40°C in the
presence of 15 ul of protein G plus protein A agarose and
1M NaCl adjusted to pH 8.0. After several washes with 20
15   mM Tris HCl, pH 8.0, 1M NaCl, 5mM MgCl$_2$, 0.2% Triton X-100
and 1 mM PMSF, immunoprecipitates were separated over 8%
PAGE and subjected to autoradiography. A band of ~ 0 kD
molecular weight was present only in lysates
immunoprecipitated with anti p75^{NTR} antibodies and not
20   lysates immunoprecipitated with irrelevant mouse IgG.

        p75^{NTR}-NIH 3T3 cells were incubated for 2 hours at 40°C
in binding medium (DMEM, 10 mM hepes, 0.1 mg/ml cytochrome
C, 0.01% Tween 80, 1 mg/ml BSA) with ^{125}I β-amyloid 1-40 and
increasing concentrations of NGF (0-100 ng/ml). After
25   rinsing in PBS cells were lysed in 1N NaOH and equal
amounts of protein from cell lysates were subject to γ
counting. A concentration dependent inhibition of ^{125}I β-
amyloid binding by NGF was observed with a maximum of 38%
inhibition at NGF concentrations of 100 ng/ml and
30   statistically comparable binding at 25 ng/ml.

EXAMPLE 6: CYCLIC PEPTIDE COMPETITIVELY INHIBITS β-AMYLOID
BINDING TO p75^{NTR}

        p75^{NTR} 3T3 cells were incubated in suspension at 4°C
35   for 4 hours with 0.5 uCi^{125}I β-amyloid 1-40 and increasing

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concentrations (0-400 nM) of the cyclic peptide CVGSNKGAIC
(SEQ ID NO: 4). Lysates from 1.5 X 10^5 cells were
subjected to counting. As shown in Figure 1, concentration
dependent inhibition of ^{125}Iβ-amyloid 1-40 binding by the
5   cyclic peptide was observed with 50% inhibition was
observed at the expected 25 nM cyclic peptide
concentration. This experiment demonstrates the cyclic
peptide can compete with β-amyloid 1-40 for binding to the
p75^{NTR} receptor.

10

EXAMPLE 7: EFFECT OF PEPTIDE ON CELL SURVIVAL
        p75^{NTR} 3T3 cells were maintained in serum free medium
supplemented with 200 nM β-amyloid 1-40, 200 nM cyclic
peptide CVGSNKGAIC, (SEQ ID NO: 4) 20 nM β-amyloid 1-40 and
15   200 nM cyclic peptide or diluent alone. Cell yields
determined up to 120 hours after addition of β-amyloid show
prominent decrease of cell yield in cultures maintained
with 200 nM β-amyloid 1-40 as compared to all other
cultures (see Figure 2).

20

EQUIVALENTS
        Those skilled in the art will recognize, or be able to
ascertain using no more than routine experimentation, many
equivalents to the specific embodiments of the invention
25   described herein. Such equivalents are intended to be
encompassed by the following claims.

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<u>CLAIMS</u>

The invention as claimed is:

1.   A method of evaluating the risk of an individual to
      develop Alzheimer's disease associated with β-amyloid
      protein or β-amyloid peptide activation of the p75
      nerve growth factor receptor, comprising the steps of:
      a)   culturing growing epidermal melanocytes
            expressing the p75 nerve growth factor receptor
            obtained from the individual;
      b)   introducing β-amyloid protein or β-amyloid
            peptide into the melanocyte culture of step a) in
            a concentration sufficient for the β-amyloid
            protein or peptide to activate the p75 nerve
            growth factor receptor;
      c)   determining whether the p75 nerve growth factor
            receptor is activated by β-amyloid protein or β-
            amyloid peptide; and
      d)   comparing the activation of the p75 nerve growth
            factor receptor of melanocytes cultured in step
            b) with the activation of the p75 nerve growth
            factor receptor of control melanocytes also
            cultured in the presence of β-amyloid protein or
            peptide, wherein activation of the p75 nerve
            growth factor receptor of the melanocytes of step
            b) greater than activation of p75 nerve growth
            factor receptor in the control melanocytes is an
            indication that the individual is at risk to
            develop Alzheimer's disease.

2.   A method according to Claim 1, wherein determining
      whether the p75 nerve growth factor receptor is
      activated further comprises an assay method selected

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from the group consisting of: an assay to determine melanocyte cell yield, an assay to determine the inducement of Bax protein expression; an assay to determine the onset of melanocyte apoptosis or an assay to determine the presence of plaque-like structures in the melanocyte culture.

3. A method according to Claim 2, wherein the assay to determine the onset of melanocyte apoptosis is an assay selected from the group consisting of: measurement of propiduim iodine incorporation into nuclear fragments, the TUNEL reaction or demonstration of fragmented DNA.

4. A method according to Claim 1, wherein the β-amyloid peptide is a peptide selected from the group consisting of: β-amyloid 1-40 peptide; β-amyloid 1-42 peptide; β-amyloid 25-36 peptide or β-amyloid 28-30 peptide.

5. A method according to Claim 1, wherein the β-amyloid protein is β-amyloid precursor protein.

6. A method according Claim 1, wherein the concentration of β-amyloid protein or β-amyloid peptide in step b) is from about 1 μM to about 100 μM.

7. A method according to Claim 1, wherein in step a) a series of multiple cultures of the melanocytes is maintained; in step b) β-amyloid protein or β-amyloid peptide is added to each culture such that each culture in the series receives an increasing concentration of β-amyloid protein or β-amyloid peptide ranging from about 0 μM to about 100 μM and in step c) the activation of the p75 nerve growth factor

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receptor is determined and correlated to the concentration of β-amyloid protein peptide added.

8. A method of evaluating the risk of an individual to develop Alzheimer's disease associated with β-amyloid protein or β-amyloid peptide activation of the p75 nerve growth factor receptor, comprising the steps of:

    a) culturing epidermal melanocytes expressing the p75 nerve growth factor receptor obtained from the individual, thereby producing a test culture, and culturing a control cell line of epidermal melanocytes expressing the p75 nerve growth factor receptor, thereby producing a control culture, under conditions suitable for maintaining the melanocyte cultures;

    b) determining the amount of β-amyloid protein or β-amyloid precursor protein produced by the test culture and control culture; and

    c) comparing the amount of β-amyloid precursor protein or β-amyloid proteins produced, wherein production of the β-amyloid precursor protein, or protein in the test culture greater than production of β-amyloid precursor protein, or peptide on the control culture is an indication that the individual is at risk for Alzheimer's disease.

9. A method according to Claim 8, wherein the production of β-amyloid precursor protein or β-amyloid protein is determined by Northern blot analysis to measure the amount of β-amyloid precursor protein mRNA, or β-amyloid protein mRNA expressed in the melanocytes.

10. A method according to Claim 8, wherein the production of β-amyloid precursor protein or β-amyloid protein is

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determined by Western blot analysis using an antibody specific for the β-amyloid precursor protein or β-amyloid protein.

5   11.   A method according to Claim 8, wherein after step a), the cultures are exposed to UV irradiation.

     12.   A method of evaluating the risk of an individual to develop Alzheimer's disease associated with β-amyloid
10   protein or β-amyloid peptide activation of the p75 nerve growth factor receptor, comprising the steps of:
             a)   culturing epidermal melanocytes obtained from the individual;
             b)   measuring the amount of p75 nerve growth factor
15   and p140 tyrosine kinase A receptor expressed on the surface of the cultured melanocytes; and
             c)   determining the ratio of the amount of p75 nerve growth factor expressed relative to the amount of p140 tyrosine kinase A expressed.

20
     13.   A method of treating Alzheimer's disease in an individual, comprising inhibiting the binding of β-amyloid protein or β-amyloid peptide to the p75 nerve growth factor receptor expressed on central nervous
25   system neuronal cells, comprising administering to the individual a peptide comprising the amino acid sequence lysine-glycine-alanine, whereby the peptide binds to the p75 nerve growth factor receptor and inhibits the binding of β-amyloid protein or peptide
30   to the p75 nerve growth factor receptor.

     14.   A method according to Claim 13 wherein the peptide is SEQ ID NO: 4.

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15. A method of decreasing the risk of β-amyloid protein-mediated apoptosis of neuronal cells comprising, inhibiting the binding of β-amyloid protein or β-amyloid peptide to the p75 nerve growth factor receptor expressed on central nervous system neuronal cells, comprising administering to the vertebrate a peptide comprising the amino acid sequence lysine-glycine-alanine whereby the peptide binds to the p75 nerve growth factor receptor and inhibits the binding of β-amyloid protein or peptide to the p75 nerve growth factor receptor.

16. A method according to Claim 15 wherein the peptide is SEQ ID NO: 4.

17. Use of a peptide comprising the amino acid sequence lysine-glycine-alanine, which peptide binds to the p75 nerve growth factor receptor and inhibits the binding of β-amyloid protein or β-amyloid peptide to the p75 nerve growth factor receptor expressed on central nervous system neuronal cells, for the manufacture of a medicament for treating Alzheimer's disease in an individual

18. Use for the manufacture of a medicament for decreasing the risk of β-amyloid protein-mediated apoptosis of neuronal cells of a peptide comprising the amino acid sequence lysine-glycine-alanine which peptide binds to the p75 nerve growth factor receptor and inhibits the binding of β-amyloid protein or peptide to the p75 nerve growth factor receptor expressed on central nervous system neuronal cells.

19. A peptide for use in therapy, e.g. the treatment of
Alzheimer's disease, comprising the amino acid
sequence lysine-glycine-alanine, which peptide binds
to the p75 nerve growth factor receptor and inhibits
the binding of β-amyloid protein or peptide to the p75
nerve growth factor receptor.

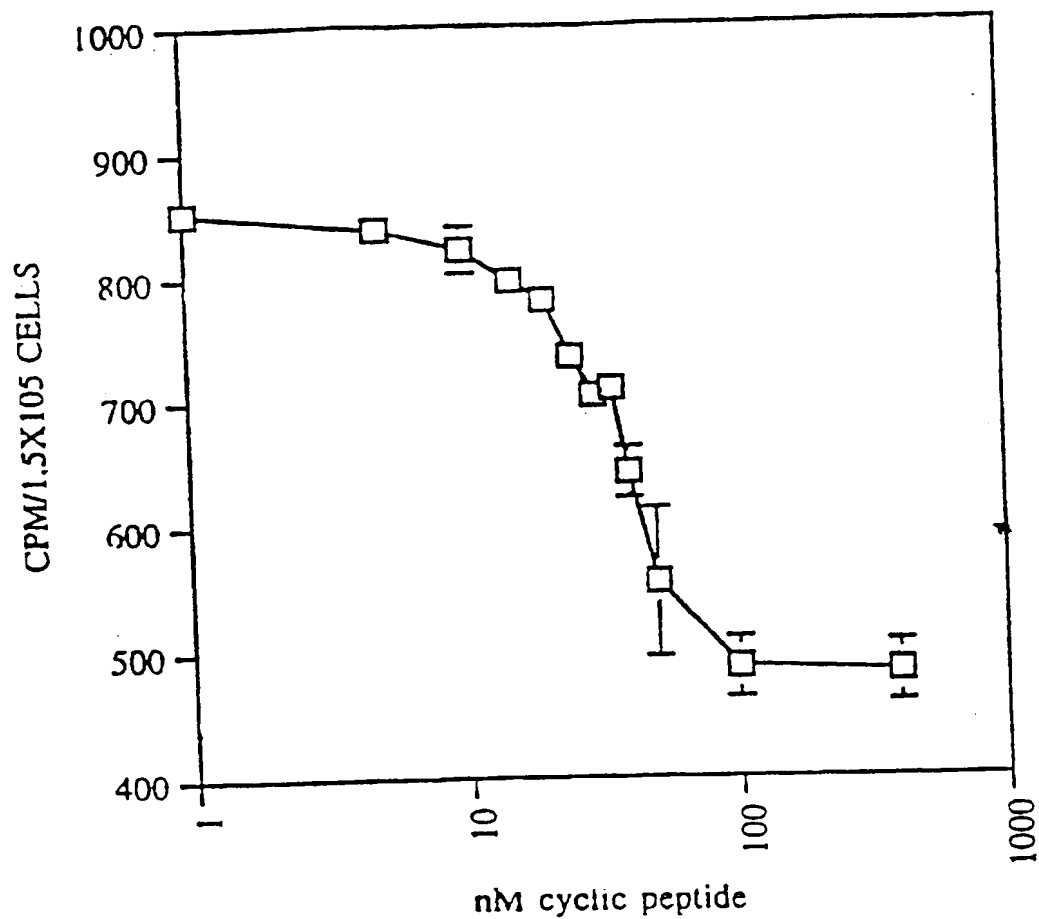THE CYCLIC PEPTIDE COMPWTITIVELY INHIBIT β AMYLOID BINDING TO P75

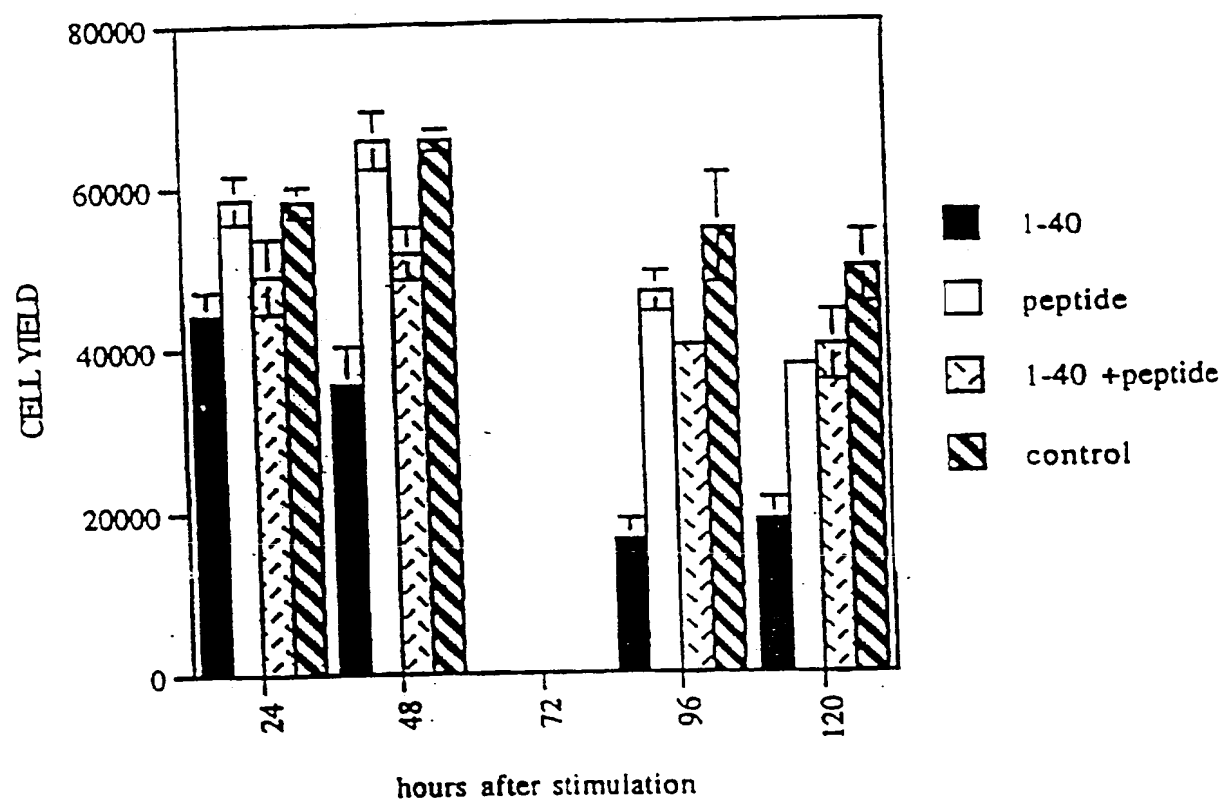FIGURE 1

EFFECT OF CVGSWNKGAIC ON P75 3T3 CELL SURVIVAL

FIGURE 2

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   G01N33/68   G01N33/567   C07K14/47   C07K14/48   A61K38/17
          A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF CLINICAL INVESTIGATION, vol. 94, no. 4, 1 October 1994, pages 1550-1562, XP000560963 YAAR M ET AL: "THE TRK FAMILY OF RECEPTORS MEDIATES NERVE GROWTH FACTOR AND NEUROTROPHIN-3 EFFECTS IN MELANOCYTES" cited in the application see abstract ---	1
A	WO 92 18149 A (REGENERON PHARMACEUTICALS INC) 29 October 1992 see abstract ---	1,13
A	WO 95 21193 A (UNIV MCGILL ;SARAGOVI URI H (CA); LESAUTEUR LYNNE (CA); CUELLO A C) 10 August 1995 see the whole document ---	12-19

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 August 1997	28.08.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl. Fax (+ 31-70) 340-3016	Ceder, 0

Form PCT ISA 210 (second sheet) (July 1992)

International Application No

PCT/US 97/04966

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 584 452 A (AMERICAN CYANAMID CO) 2 March 1994 see Sequence listing SEQ ID NO: 2: amino acids 412-419 ---	19
A	EXPERIMENTAL NEUROLOGY, vol. 124, 1993, NEW YORK, pages 16-20, XP002037880 LAPCHAK: "Nerve growth factor pharmacology: Application to the treatment of cholinergic neurodegeneration in Alzheimer's disaese" cited in the application see page 18, right-hand column ---	13-19
P,X	WO 96 12955 A (UNIV CALIFORNIA) 2 May 1996 see the whole document -----	13,15, 17,18

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INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
   > Remark: Although claim(s) 13-16
   > is(are) directed to a method of treatment of the human/animal
   > body, the search has been carried out and based on the alleged
   > effects of the compound/composition.

2. ☐ Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                               ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9218149 A	29-10-92	AU 676664 B	20-03-97
		AU 2014992 A	17-11-92
		CA 2109100 A	29-10-92
		CZ 9302237 A	17-08-94
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		HU 66051 A	28-09-94
		JP 6509413 T	20-10-94
		NO 933819 A	20-12-93
		NZ 242468 A	22-12-94
		SK 116593 A	09-11-94
		US 5622862 A	22-04-97
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		EP 0743955 A	27-11-96
EP 0584452 A	02-03-94	AU 3835893 A	04-11-93
		CA 2095421 A	02-11-93
		JP 7132094 A	23-05-95
		NZ 247499 A	26-04-96
		US 5652092 A	29-07-97
		ZA 9303084 A	24-11-93
WO 9612955 A	02-05-96	US 5576209 A	19-11-96
		AU 4005195 A	15-05-96